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ORGANIC OSMOLYTES AND CELL VOLUME REGULATION

Transcriptional responses to tubule challenges

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Transcriptional responses to tubule challenges. Adaptation to physiological stimuli often involves changes in gene transcription. Studies of hyperosmolar stress in renal epithelial cells have provided an ideal paradigm for understanding regulation of gene expression. Renal epithelial cells respond very differently to hyperosmolar NaCl and urea and several strategies including cloning based on known biological function, candidate gene analysis, and differential display analysis have successfully identified many genes induced by these hyperosmolar challenges. Hyperosmolar NaCl produces adverse effects on cellular biosynthetic processes and compensatory increases are observed in transcription of transporters, stress proteins, and metabolic enzymes. In contrast, hyperosmolar urea fails to inhibit biosynthetic processes but, nonetheless, initiates a very specific program of gene expression in renal epithelial cells. This program appears to involve a urea sensor/receptor system which activates transcription and translation of the zinc-finger transcription factor *Egr-1*. This work highlights the concept that rapid analysis of differential gene expression will enable one to define cellular programs of gene expression involving up- and down-regulation of functionally-related gene families.

The kidney is capable of maintaining body fluid and solute homeostasis under a wide range of environmental conditions. Adaptation of the renal tubule epithelial cells to various physiological and pathophysiological challenges involves alterations in gene expression related to transcription and translation as well as post-translational alterations in the activities of proteins. Relatively recent advances in molecular biology have enabled investigators to begin to define the array of genes whose expression is altered in kidney epithelia during various challenges. Herein we briefly summarize current approaches that are available to define the genetic programs used by renal epithelia to adapt to various stimuli. In this context, we will describe our efforts to identify functionally-related gene families that are over-expressed during adaptation of renal epithelial cells to hyperosmotic stress.

The hyperosmolar renal medulla–A harsh environment

Cells of the renal medulla are exposed to extreme variations in osmolality ranging from less than 100 mOsm during maximal diluting conditions to nearly 3000 mOsm during water deprivation in laboratory rats. These extraordinary shifts in NaCl and urea concentrations cause profound changes in cellular functions. The adverse effects of hyperosmolar stress include cell shrinkage, altered levels of intracellular ions, inhibition of enzyme activities, inhibition of biosynthetic processes, and, in extreme states, denaturation of proteins [1, 2]. The ability to survive this environment is a specialized feature of renal medullary cells that involves a specialized program of signal transduction, transcription of a specific subset of genes, and cellular actions of the effector genes that enable cells to adapt to the hyperosmotic milieu.

To elucidate the program of events involved in adaptation to hyperosmolar stress, several groups have investigated the effects of hyperosmolar NaCl and urea on cultured renal epithelial cells [3]. Of particular note, it was found that hyperosmolar (+200 to 400 mOsm) NaCl and urea have very different effects on renal epithelial cells. Hyperosmolar NaCl inhibited numerous biosynthetic processes including synthesis of protein, RNA and DNA [1]. In contrast, hyperosmolar urea failed to inhibit these processes and, in fact, was shown to stimulate DNA synthesis in MDCK and LLC-PK_1 cells [1, 4]. Moreover, hyperosmolar NaCl was found to stimulate cellular accumulation of organic osmolytes such as betaine, sorbitol, inositol, glycerophosphorylcholine (GPC), and taurine whereas hyperosmolar urea increased GPC levels only [3]. These observations have led to the concept that hyperosmolar NaCl and urea initiate two distinctly different programs of gene expression. Characterization of these two programs has involved an intense search for those genes that are overexpressed under these two conditions.

Hunting for osmotic stress-induced genes

The hunt for genes whose expression is elevated during hyperosmolar NaCl or urea stress has been a particularly tedious process because of problems inherent to the search process. In particular, there are estimated to be 100,000 expressed genes in mammals of which approximately 10,000 to 15,000 are expressed in any given cell. Distinguishing which of these thousands of genes is responsive to hyperosmotic stress has involved three basic approaches (Table 1): cloning based on known biological function, the candidate gene approach, and differential gene expression analysis.

As described elsewhere in this symposium, cloning based on known biological function has been a highly successful approach that has been used by Drs. Burg, Handler, Garcia-Perez, Kwon and their coworkers [5, 6]. These investigators established the molecular identity of the cDNAs responsible for synthesis or uptake of organic osmolytes including sorbitol, *myo*-inositol, betaine, and taurine. Hyperosmolar NaCl was shown to enhance transcription of these genes yielding an order of magnitude increase in mRNA levels that peaked at approximately 18 to 24 hours after the onset of the stimulus. While highly successful in finding osmotic stress-induced genes, this approach has the limitation that the investigator must be aware of a biological function

Table 1. Strategies for identifying osmotic stress-induced genes

- Cloning based on known biological function
- Candidate gene approach
- Differential gene expression analysis

Table 2. Approaches to differential gene expression analysis

- 2-D protein gel electrophoresis
- Subtraction cloning
- Comparative EST analysis
- Differential display analysis

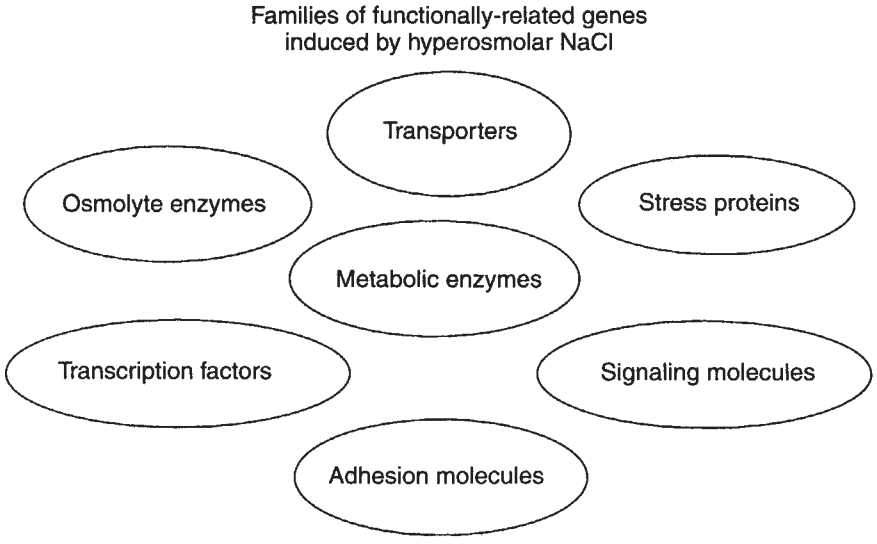

Fig. 1. *Families of functionally-related genes induced by hyperosmolar NaCl.*

that is induced and must have an assay suitable for isolating the gene that is responsible for this function.

The candidate gene approach has been equally successful in elucidating genes whose expression is augmented by hyperosmotic stress. By surveying the literature it has been possible to take an educated guess as to which genes are likely to be transcriptionally activated by hyperosmotic stress. For example, transporters such as the Na,K-ATPase and Na/H-exchanger were shown to be overexpressed during hyperosmotic stress [7, 8]. In addition, based on prior knowledge of their roles in adapting to other stress states, stress proteins such as Hsp70, BiP, and GRP95, also known as molecular chaperones, were found to be induced by hyperosmolar NaCl stress but not hyperosmolar urea [1, 9, 10]. The candidate gene approach also led to the observation that mRNA levels of transcription factors such as *Egr-1* and c-*fos* [1] as well as several known signaling molecules are up-regulated by hyperosmolar NaCl.

The approaches described above provided a firm understanding of the families of functionally-related genes that are induced by hyperosmolar NaCl (Fig. 1). These include inorganic and organic solute transporters, organic osmolyte synthesizing enzymes, stress proteins, transcription factors, and signaling molecules. In fact, the relatively rapid success of using the candidate gene approach has been sufficiently encouraging that it is reasonable to expect this approach to continue to yield valuable discoveries in the future. In this vein, it is provocative to consider the enormous impact that will be felt from the Human Genome Project, particularly the current effort to sequence all 100,000 expressed genes. Pioneered by Dr. Venter and coworkers, the Expressed Sequence Tag (EST) databases have already provided more than 50 million base pairs of DNA sequence from tens of thousands of expressed mRNAs. Within these databases are numerous new genes with homologies to known gene families, as well as genes with completely novel structures whose biological functions remain to be elucidated. As we look forward, it is both exciting and daunting to consider how we will incorporate this new information into our search for genes responsible for adaptation to osmotic stress and other physiological stimuli.

Differential gene expression analysis

To acquire a complete evaluation of the osmotically responsive genes in renal epithelia, it will be necessary to circumvent the biased approach inherent in candidate gene analysis or cloning based on known biological function. Accordingly, several relatively unbiased methods are available for identifying and cloning differentially expressed genes. In general, protein or mRNA is isolated from control and stimulated cells and subjected to a differential screening process to identify differentially expressed products. As indicated in Table 2, these analytical procedures include two-dimensional (2-D) protein gel electrophoresis, subtraction cloning and related methods, comparative EST analysis, and differential display analysis. All these methods are potentially valuable, however, our experience has shown that differential display analysis is perhaps the most valuable because of a combination of sensitivity, reliability, cost-effectiveness, and efficiency.

Differential display analysis of mRNA expression was developed independently by Liang and Pardee [11] and Welsh and McClelland [12]. Briefly, this method involves the use of arbitrary oligonucleotide primers in combination with RT-PCR to amplify and compare the expression levels of 20 to 100 mRNAs. An example of this method is shown in Figure 2 in which RNA was isolated from a variety of tissues of normonatremic and hypernatremic rats and compared side-by-side with differential display analysis. Changes in mRNA expression are evident by differing

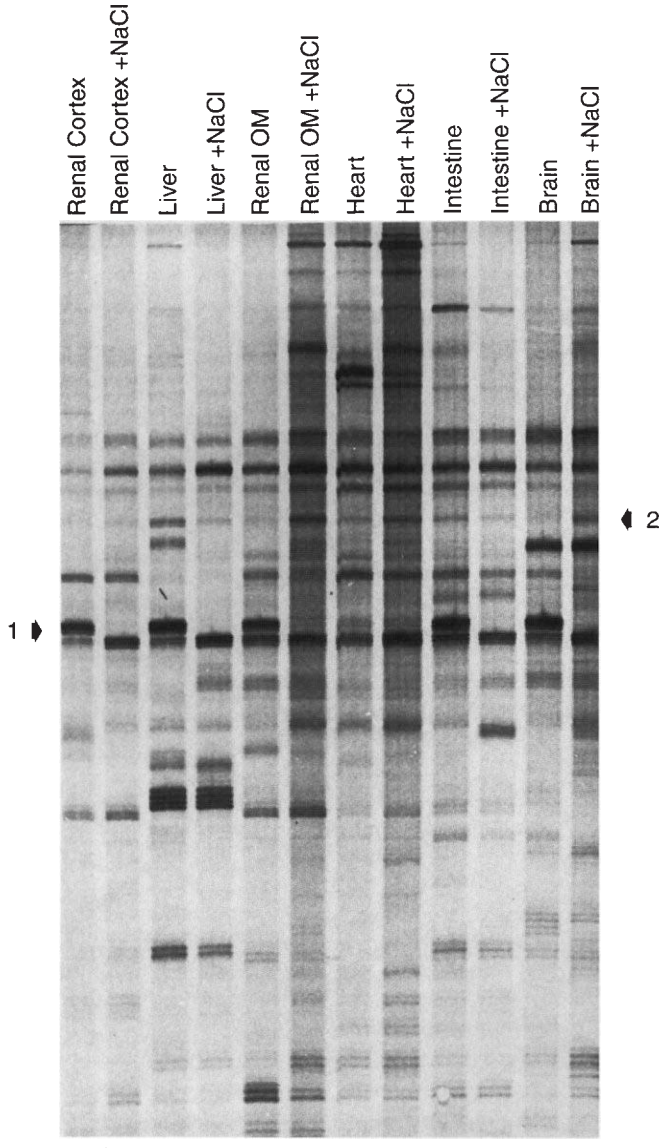

Fig. 2. *Differential display analysis of mRNA expression in tissues derived from normonatremic and hypernatremic (+NaCl) rats.* Each lane displays approximately 20 to 30 different cDNAs. Rats were treated as described previously [19]. The arrows indicate an mRNA that is either suppressed (arrow 1) or induced (arrow 2) in the hypernatremic rats. OM, renal outer medulla.

intensities of signal from individual bands on the autoradiograph. Up- or down-modulated cDNAs are then cut from the gel, cloned, and sequenced providing a rapid analysis of the differentially expressed mRNAs.

We have used differential display analysis to identify genes whose expression was up-regulated at the mRNA level by hyperosmotic NaCl in mIMCD3 cells. After examining approximately 3000 mRNAs we isolated, cloned, and sequenced 11 cDNA products. Of these seven were known genes and four were novel [13]. When examined using northern analysis, we confirmed that 10 of 11 were up-regulated with hyperosmotic NaCl stress. The known genes included enzymes of intermediary metabolism, transporters, a transcription factor, and an adhesion molecule. Of the novel genes, one showed significant sequence homology to the Hsp70 stress protein family. We subsequently cloned and sequenced the full-length cDNA of this stress protein and named it osmotic stress protein 94 or Osp94 [14]. The deduced amino acid sequence showed it is a 94 kD molecular chaperone with both an ATP-binding domain and a putative peptide-binding domain. Comparison of Osp94 to the nucleotide and protein databases confirmed that Osp94 is related to the heat shock protein 70 family, and further indicated that it is a member of a previously unrecognized subfamily of this gene family. This subfamily of genes also includes the recently cloned Hsp110 and Hsp70RY cDNAs. Northern analysis indicated that Osp94 is transiently up-regulated many-fold during exposure to hyperosmotic NaCl stress. Interestingly, Hsp110 but not Hsp70RY was also up-regulated at the mRNA level with hyperosmotic NaCl stress. The function of Osp94 and the other members of this stress protein sub-family remains to be elucidated but, because of their structural similarity to Hsp70 and BiP, they are likely to be involved in the process of protein synthesis and folding.

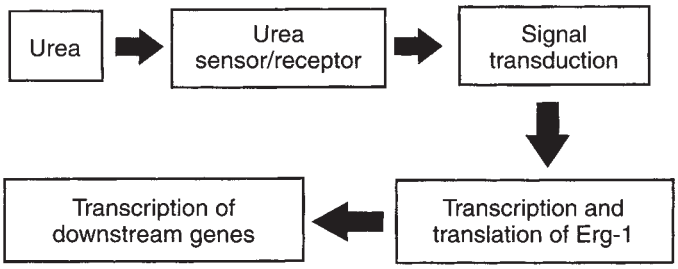

Fig. 3. *Proposed actions of urea in renal epithelial cells.* Hyperosmolar urea, acting via a urea sensor/receptor initiates a signal transduction process that stimulates transcription of *Egr-1* and other transcription factors. This, in turn, activates transcription of downstream target genes.

Table 3. Urea responsive cells

Cell type	Renal epithelial cell	*Egr-1* induced
MDCK	Yes	Yes
LLC-PK$_1$	Yes	Yes
mIMCD3	Yes	Yes
bMES	No	No
T84	No	No
BAE	No	No
C6-glioma	No	No

A total of 200 mM urea was added to each cell type and Egr-1 mRNA expression was analyzed. Abbreviations are: bMES, bovine mesangial endothelial cells; BAE, bovine aortic endothelial cells.

Thus, using a variety of gene hunting strategies it has been shown that renal epithelial cells overexpress a selected subset of genes that enable them to adapt to and survive hyperosmolar NaCl stress. The genes that are recruited during this adaptation process encode functionally related proteins whose functions include solute transport, energy metabolism, cell adhesion, signal transduction, and protein synthesis.

Hyperosmolar urea activates transcription in renal epithelia

In recent years work from our laboratory has provided evidence that urea is a potent "factor" for activating gene transcription in renal epithelial cells [4, 15, 16]. Following an initial observation that hyperosmolar urea rapidly and transiently increases mRNA

levels of the transcription factors *Egr-1* and c-*fos* in MDCK cells, we examined the effects of urea on *Egr-1* in a variety of cell types. As shown in Table 3, urea increased *Egr-1* mRNA expression only in cell lines that were derived from renal epithelia; neither non-renal epithelial cells nor non-epithelial renal cells responded. Moreover, it was established that this effect was very specific for urea in the concentration range of approximately 50 to 400 mM. Neither the permeant solute glycerol nor the poorly permeant solute mannitol mimicked the effect of urea. In addition, urea analogues including N-methylurea, 1,1-dimethylurea, 1,3-dimethylurea, thiourea and acetamide failed to mimic the ability of urea to activate *Egr-1* transcription. Subsequent studies have firmly established that urea rapidly increases transcription and translation of *Egr-1* and that *Egr-1* protein can act as a functional transcription factor [16]. Interestingly, urea was shown to be equipotent or even more potent than a variety of growth factors as well as serum in activating *Egr-1*. More recent studies have begun to elucidate the urea responsive promoter elements in the *Egr-1* gene as well as define a specific MAP kinase signal transduction process which is involved [17].

It is readily apparent that hyperosmolar urea initiates a program of events in renal epithelial cells which is entirely different from that observed with hyperosmolar NaCl. The failure of urea (< 400 mM) to adversely affect cellular functions or increase expression of stress proteins indicates that the genetic response is not that of a stressed cell. Rather, it appears that urea activates an alternative program of gene transcription in renal epithelial cells. Initial studies with MDCK and LLC-PK_1 cells showed urea increases DNA synthesis hinting that it might act as a mitogen [18]. However, cell proliferation was not evident. Our current hypothesis regarding urea activation of gene transcription is that it may represent one facet of the hypertrophy response observed in kidney following high protein diet but further studies will be needed to examine this issue.

In an effort to define the downstream events resulting from urea-activation of renal epithelial cells we examined several known targets of *Egr-1* and none was increased at the mRNA level. More recently we began to use differential display to identify urea-inducible genes at early and late time points in mIMCD3 cells. Thus far several urea-inducible mRNAs have been identified and include both novel and known genes. Of particular note, one of the genes that was isolated was *Egr-1*, an observation that strongly validated our ability to identify urea-inducible mRNAs using this methodology.

Conclusion

Hyperosmolality is a potent stimulus for gene transcription in renal epithelial cells. In recent years it has become apparent that renal epithelial cells are programmed to adapt to hyperosmotic NaCl and urea in very different ways. In the case of NaCl, the cells increase expression of transporters, metabolic enzymes, stress proteins and other related proteins. In contrast, urea fails to activate transcription of any of these genes but rather activates, via a urea-specific signal transduction mechanism, the expression of a different subset of genes that remains incompletely characterized. Through the analysis of differential gene expression using methods such as differential display we anticipate that these separate programs of gene activation will be more fully elucidated.

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